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Assembly and budding of influenza virus

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Abstract

Influenza viruses are causative agents of an acute febrile respiratory disease called influenza (commonly known as "flu") and belong to the Orthomyxoviridae family. These viruses possess segmented, negative stranded RNA genomes (vRNA) and are enveloped, usually spherical and bud from the plasma membrane (more specifically, the apical plasma membrane of polarized epithelial cells). Complete virus particles, therefore, are not found inside infected cells. Virus particles consist of three major subviral components, namely the viral envelope, matrix protein (M1), and core (viral ribonucleocapsid [vRNP]). The viral envelope surrounding the vRNP consists of a lipid bilayer containing spikes composed of viral glycoproteins (HA, NA, and M2) on the outer side and M1 on the inner side. Viral lipids, derived from the host plasma membrane, are selectively enriched in cholesterol and glycosphingolipids. M1 forms the bridge between the viral envelope and the core. The viral core consists of helical vRNP containing vRNA (minus strand) and NP along with minor amounts of NEP and polymerase complex (PA, PB1, and PB2). For viral morphogenesis to occur, all three viral components, namely the viral envelope (containing lipids and transmembrane proteins), M1, and the vRNP must be brought to the assembly site, i.e. the apical plasma membrane in polarized epithelial cells. Finally, buds must be formed at the assembly site and virus particles released with the closure of buds.

Transmembrane viral proteins are transported to the assembly site on the plasma membrane via the exocytic pathway. Both HA and NA possess apical sorting signals and use lipid rafts for cell surface transport and apical sorting. These lipid rafts are enriched in cholesterol, glycosphingolipids and are relatively resistant to neutral detergent extraction at low temperature. M1 is synthesized on free cytosolic polyribosomes. vRNPs are made inside the host nucleus and are exported into the cytoplasm through the nuclear pore with the help of M1 and NEP. How M1 and vRNPs are directed to the assembly site on the plasma membrane remains unclear. The likely possibilities are that they use a piggy-back mechanism on viral glycoproteins or cytoskeletal elements. Alternatively, they may possess apical determinants or diffuse to the assembly site, or a combination of these pathways. Interactions of M1 with M1, M1 with vRNP, and M1 with HA and NA facilitate concentration of viral components and exclusion of host proteins from the budding site. M1 interacts with the cytoplasmic tail (CT) and transmembrane domain (TMD) of glycoproteins, and thereby functions as a bridge between the viral envelope and vRNP.

Lipid rafts function as microdomains for concentrating viral glycoproteins and may serve as a platform for virus budding. Virus bud formation requires membrane bending at the budding site. A combination of factors including concentration of and interaction among viral components, increased viscosity and asymmetry of the lipid bilayer of the lipid raft as well as pulling and pushing forces of viral and host components are likely to cause outward curvature of the plasma membrane at the assembly site leading to bud formation. Eventually, virus release requires completion of the bud due to fusion of the apposing membranes, leading to the closure of the bud, separation of the virus particle from the host plasma membrane and release of the virus particle into the extracellular environment. Among the viral components, M1 contains an L domain motif and plays a critical role in budding. Bud completion requires not only viral components but also host components. However, how host components facilitate bud completion remains unclear. In addition to bud completion, influenza virus requires NA to release virus particles from sialic acid residues on the cell surface and spread from cell to cell. Elucidation of both viral and host factors involved in viral morphogenesis and budding may lead to the development of drugs interfering with the steps of viral morphogenesis and in disease progression.

Keywords: Influenza; Matrix protein; Viral ribonucleocapsid; Virus budding; Virus assembly

1. Introduction

Assembly and budding of virus particles are the last but critically important steps in the virus life cycle for both the

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survival of the virus as well as its disease-producing ability in the host. Without completion of these two steps, infected cells will undergo abortive infectious cycles without releasing the complete virus particles. Specific host or tissue cells may provide restrictions at multiple steps of the virus life cycle including binding, entry, uncoating, synthesis and transport of viral components as well as assembly and budding. Each of these steps is an important target for antiviral prophylaxis and therapy. Furthermore, since a host is usually infected at a very low multiplicity of infection (MOI), an efficient multicycle replication including virus release and infection of new cells is obligatory for virus survival and pathogenesis. In addition, the site of virus budding may determine, at least partially, the nature of viral diseases. For example, most viruses causing viremia and systemic disease usually bud from the basolateral surface or cause cell-to-cell transmission by cell fusion forming heterokaryons. These viruses are usually pantropic and can infect multiple internal organs. On the other hand, viruses like influenza virus, which bud apically and cannot cause cell-to-cell fusion, are usually restricted to lungs and are pneumotropic in mammals.

Influenza viruses are negative stranded, segmented, enveloped RNA viruses containing helical ribonucleocapsid (also called viral ribonucleoprotein [vRNP]) and belong to the *Orthomyxoviridae* family (Lamb and Krug, 2001). Virus particles are usually spherical and approximately 100 nm in diameter (Fujiyoshi et al., 1994). The viral envelope consists of a lipid bilayer containing transmembrane proteins on the outside and matrix protein (M1) on the inside. Lipids are derived from the host plasma membrane but are selectively enriched in cholesterol and glycosphingolipids (Scheiffele et al., 1999; Zhang et al., 2000). Three transmembrane envelope proteins (hemagglutinin [HA], neuraminidase [NA], and M2 [ion channel]) are anchored in the lipid bilayer of the viral envelope. HA, a type I transmembrane protein, is a homotrimer and is the major envelope protein (∼80%) forming the spikes. HA provides the receptor-binding site and elicits neutralizing antibodies. Cleavage of HA is essential for fusion and virus infectivity. NA, a type II transmembrane protein, is present as a homotetramer on the viral envelope. NA removes the cell surface receptor (sialic acid) and is critical for the release of virus particles from the cell surface and spread of virus. M2, a type III transmembrane protein, is a minor protein component (only 16–20 molecules/virion) of the viral envelope. M2 is a homotetramer, functions as an ion channel (for review, see Lear, 2003; Wu and Voth, 2003), and is crucial during uncoating for dissociating the vRNP from M1 in the early phase of the infectious cycle.

The viral core consists of helical ribonucleocapsids (i.e. vRNP) containing vRNA (negative stranded) and NP along with minor amounts of the nuclear export protein (NEP) (formerly called non-structural protein NS2) and three polymerase (3P) proteins (PB1, PB2, PA) which form the viral RNA polymerase complex (3P complex) (for review, see Elton et al., 2002; Portela and Digard, 2002).

Influenza virus particles bind to cell surface sialic acid, ubiquitously present on glycoproteins or glycolipids. The specificity of the sialic acid (α2,3-linked or α2,6-linked sialic acid) and preferred binding of a particular strain of influenza virus to a specific sialic acid receptor are important determinants for species-specific restriction of influenza viruses (for review, see Matrosovich and Klenk, 2003).

During the infectious cycle, virus particles, bound to cell surface sialic acid, are internalized by receptor-mediated endocytosis and viruses possessing cleaved HA undergo fusion with the endosomal membrane (for review, see Skehel and Wiley, 2000; Stegmann, 2000) at low pH (pH ∼5.0). Cleavage of HA is an absolute requirement for infectivity and the nature of the HA cleavage site is an important virulence determinant for influenza viruses. Cleavage efficiency of HA varies depending on the presence of single or multiple basic residues at the cleavage site of HA1 and HA2 and the plasminogen binding ability of NA. Viruses containing HA with a single positive charge at the cleavage site can be cleaved by specific enzymes such as *tryptase Clara* present in the lungs, whereas HA containing multiple basic residues at the cleavage site are cleaved ubiquitously by proteases (for review, see Kido et al., 1999a). WSN NA binds plasminogen which when converted to plasmin can cleave HA even with a single basic residue (Goto and Kawaoka, 1998). In the acid pH of the endosome, the cleaved HA undergoes conformational changes releasing the NH_2 terminal fusion peptide of HA2 and causing fusion of viral and endosomal membranes (for review, see Colman and Lawrence, 2003). Virus particles containing uncleaved HA can bind and be endocytosed but cannot undergo fusion and are therefore non-infectious. The M2 ion channel opens up in the acidic pH of the endosome, acidifies the internal virion core, and thereby facilitates the release of vRNP from M1 into the cell cytoplasm. M1-free vRNP is then imported into the nucleus through nuclear pores using nuclear transport signals of NP (Neumann et al., 2000). Inside the nucleus, vRNP undergoes transcription (mRNA synthesis) and replication (complete positive-sense complementary RNA [cRNA], vRNA [minus strands], and vRNP synthesis) (for review, see Elton et al., 2002; Portela and Digard, 2002). Progeny vRNPs, made inside the nucleus, are exported out of the nucleus into the cytoplasm with the help of M1 and NEP (Chen and Krug, 2000). Eventually, the envelope proteins (HA, NA, M2), matrix protein (M1) and vRNP (containing vRNA minus-strand, NP, 3P proteins, and NEP) are transported to the assembly site on the plasma membrane where virus particles bud and are released into the outside environment. This review deals with the processes involved in assembly and morphogenesis of influenza viruses including vRNP exit from the nucleus, sorting, and transport of subviral components to the assembly site, interaction among the viral components as well as the process of bud formation, bud completion, and virus release.

2. Steps involved in the assembly and morphogenesis of influenza virus

Morphogenesis of influenza virus is a complex multi-step process, which involves not only nucleocapsid (vRNP) formation but also envelopment of the nucleocapsid and release of viral particles into the external environment. Assembly and morphogenesis of influenza virus require a number of obligatory steps: firstly, all viral (or subviral) components must be directed and brought to the assembly site, i.e. the plasma membrane in non-polarized cells or the apical plasma membrane in polarized epithelial cells. Secondly, all viral components must interact in an orderly fashion to assemble into infectious virions. Thirdly, interaction and concentration of subviral components at the assembly site must initiate bud formation, i.e. an outward curvature of the plasma membrane. Finally, apposing membranes at the stalk of the bud must fuse causing separation of the virus particle (bud) from the host cell and release of virions into the extracellular environment.

These sub-viral components are: (a) the viral core or vRNP containing vRNA, NP, NEP, and 3P proteins; (b) M1, forming the bridge between the envelope and vRNP; and (c) the envelope, containing the viral transmembrane proteins (HA, NA, and M2) and lipids derived from host cells. Moreover, since influenza virus transcription and replication occur in the host cell nucleus, where vRNPs are formed, the vRNPs must exit from the nucleus into the cytoplasm and be transported to the assembly site for incorporation into mature viral particles. Furthermore, influenza viruses require two additional steps in the assembly process: (i) since the genome of influenza virus is segmented, multiple vRNA/vRNP segments (eight separate segments for influenza type A and B viruses, seven segments for influenza type C viruses) must be incorporated into each infectious virus particle; (ii) after bud completion, virus particles must be released from sialic acid by NA for cell-to-cell spread and transmission.

2.1. Transport of viral components to the assembly site

Since influenza viruses assemble and bud from the plasma membrane, complete influenza virus particles are not found inside infected cells. Therefore, all subviral components must be directed to the plasma membrane and, more specifically, to the apical domain of the plasma membrane in polarized epithelial cells (bronchial and lung epithelia of infected hosts and MDCK cells in culture).

2.1.1. Exit of vRNP from the nucleus of infected cells

Since vRNPs are synthesized in the nucleus, they must be exported from the nucleus into the cytoplasm and to the assembly site on the plasma membrane for envelopment and budding. Current reports give evidence that the vRNPs are exported from nucleus into cytoplasm via the cellular Crm1-mediated nuclear export pathway (Elton et al., 2001; Ma et al., 2001; Watanabe et al., 2001a) and three viral proteins including M1, NEP and NP play important roles in this process. M1 provides a critical function in the nuclear-to-cytoplasmic export of vRNP since the vRNP remains bound to nucleus in the absence of M1 (Bui et al., 2000). It has also been shown that NP and vRNP remain in the nucleus of cells infected with virus particles either lacking NS vRNA or possessing the mutant NS vRNA encoding only NS1 but not NEP protein (Neumann et al., 2000) indicating the essential role of NEP in nuclear export of vRNP. However, NEP does not interact directly with vRNPs. NEP mediates RanGTP-dependent binding to the cellular protein Crm1 via its leucine-rich nuclear export signal present at the N-terminal domain. NEP also interacts with the N-terminal domain of M1 via its C-terminal domain. An exposed tryptophan (Trp78) surrounded by a cluster of glutamate residues on NEP, and the basic nuclear localization signal (NLS) of M1, is responsible for this NEP–M1 interaction (Akarsu et al., 2003). On the other hand, M1 binds to vRNP via its C-terminal domain (Baudin et al., 2001). It was therefore suggested that a “daisy-chain” complex of (Crm1–RanGTP)–NEP–M1–vRNP mediated the export of vRNP across the nuclear envelope (Akarsu et al., 2003). However, NP alone has also been proposed to mediate vRNP export, as it interacts directly with Crm1 in vitro (Elton et al., 2001).

Several experimental observations suggest that there are likely to be two classes of vRNPs in the host cell nucleus (for review, see Nayak and Hui, 2002). One class of vRNP is involved in active transcription and replication and is not likely to be exported out of the nucleus. On the other hand, another class of vRNPs remains transcriptionally inactive, sequestered from the transcriptionally active vRNPs and is exported out of the nucleus for eventual incorporation into progeny virions. It was observed that in released progeny virus particles, polymerase molecules were present only at one end of the vRNP (Murti et al., 1988) but not all over the vRNP as would be expected if the progeny vRNPs were undergoing active transcription during their exit from the nucleus. Furthermore, dependence of in vitro mRNA synthesis on the presence of 5′ primer and the incorporation of 5′ primer into mRNA using vRNP template would also support genuine transcription initiation (Rao et al., 2003). On the other hand, a simple chain elongation, independent of primer requirement, would occur if the vRNPs were exported out of the nucleus in a transcriptionally active form and were incorporated into progeny virus particles. However, how the progeny vRNPs are rendered transcriptionally inactive in the nucleus remains unclear. Since M1 can bind to vRNA (Baudin et al., 2001; Ye et al., 1999) and inhibit transcription (Hankins et al., 1990), it is possible that a few M1 molecules can bind at the critical site on either the progeny vRNA or the viral polymerase complex and render those vRNPs transcriptionally inactive (for review, see Nayak and Hui, 2002). This hypothesis favors the model of ‘daisy-chain’ complex of (Crm1 and RanGTP)–NEP–M1–vRNP (Akarsu et al., 2003) instead of direct interaction of Crm1 and NP mediating the nuclear export of vRNPs.

2.1.2. Transport of viral envelope proteins to the apical cell surface

Among the viral components, most information is available about the transport, sorting and targeting of viral envelope proteins (HA, NA, and M2) to the virus assembly site. In virus-infected cells as well as in cells expressing envelope proteins individually from cloned cDNAs, each viral envelope protein (HA, NA, and M2) preferentially accumulates at the virus assembly site, i.e. the apical plasma membrane in polarized epithelial cells (Hughey et al., 1992; Jones et al., 1985; Roth et al., 1983). These and other studies showed that HA, NA, and M2 possess the determinants for sorting and targeting to the apical plasma membrane in polarized epithelial cells. Although the apical sorting signal of M2 is yet to be defined, the apical sorting signals for HA and NA have been studied in detail. Both HA and NA possess two apical determinants: one in the ectodomain, which is likely to be glycan, and other in the transmembrane domain (TMD). Unlike the basolateral signals, the cytoplasmic tail (CT) of either HA or NA does not contain the apical signal. Moreover, both HA and NA have been shown to interact with non-ionic detergent-resistant lipid microdomains (lipid rafts) and the determinant(s) for raft-association resides in their TMDs (Kundu et al., 1996; Lin et al., 1998). Furthermore, it has been shown that both apical (e.g. influenza HA and NA) and basolateral (e.g. vesicular stomatitis virus [VSV] G) proteins are synthesized in the same compartment of membrane-bound polyribosomes and are transported together from the ER through the *cis*-, *mid*- to *trans*-Golgi network (TGN). However, transport of the apical and basolateral proteins from the TGN to the respective plasma membrane domains occurs via separate transport vesicles (Wandinger-Ness et al., 1990).

The role of the TMD in apical sorting and lipid raft association were first demonstrated with NA, a type II transmembrane protein (Kundu et al., 1996). Further analysis of the TMD showed that signal(s) for apical transport in the TMD of NA were not present in a specific peptide sequence but extended over multiple regions of the TMD and that 19 amino acids (aa 9–27) of the NA TMD were sufficient for apical transport (Barman and Nayak, 2000). Moreover, it was also shown that the exoplasmic half of NA TMD amino acid sequences was critical for lipid raft association. However, although the determinants for both apical transport and lipid raft association resided in the NA TMD and overlapped each other, they were not identical (Barman and Nayak, 2000). Furthermore, deletion of the CT of NA caused a reduction in raft-association (Zhang et al., 2000), though the CT-minus NA protein was transported predominantly to the apical plasma membrane in polarized epithelial cells.

Mutational analysis of the amino acids in the HA TMD showed that amino acid sequences in the middle of TMD were most important for apical sorting and that the exoplasmic half of the TMD amino acid sequences were critical for lipid raft association. However, as for NA, raft-association did not completely correlate with apical transport of HA. Some mutants exhibiting high degree of raft-association were sorted randomly whereas an HA mutant transported predominantly to the apical plasma membrane without significant raft-association (Lin et al., 1998). Moreover, deletion of the CT of HA caused a reduction in raft-association (Zhang et al., 2000) without affecting its apical transport. These results again support that the determinants for apical transport and raft-association were not identical although raft-association facilitated apical sorting and both resided in the TMD of HA and NA.

2.1.3. Transport of M1 and vRNP to the assembly site

M1, the most abundant viral protein in the virus particle, plays a critical role in the processes of virion assembly and budding. For assembly and budding, both M1 and the vRNP must be transported to the assembly site. However, how M1 and the vRNP individually or jointly are transported to the budding site remains unclear. Nuclear translocation of M1 in virus-infected cells unlike that observed in cDNA-transfected cells, does not depend on the function of the M1 NLS, since a mutant M1 protein lacking an NLS can enter the nucleus when expressed with other viral components, particularly NP and vRNA (Huang et al., 2001; Perez and Donis, 1998). Neither HA nor NA is absolutely required for virus budding, since virus particles lacking either HA or NA can bud in HA or NA temperature sensitive (ts) mutants at the restrictive temperature (Palese et al., 1974; Pattnaik et al., 1986). Besides, virus budding has been shown to occur in the absence of NA (Liu et al., 1995). On the other hand, virus budding does not occur in the absence of M1, and M1 expressed alone can form virus-like particles in transfected cells (Gómez-Puertas et al., 2000; Latham and Galarza, 2001). In virus-infected polarized cells, as expected, both M1 and NP are observed at the apical plasma membrane (Mora et al., 2002). M1 and NP proteins are synthesized by the nonmembrane-bound polyribosomes and do not use the exocytic pathway for transport. Furthermore, M1 and NP proteins expressed individually or together are not transported to the apical plasma membrane (Barman and Nayak, unpublished data). Also, M1 and NP, when co-expressed, do not interact with each other in the absence of vRNP (Huang et al., 2001; Zhao et al., 1998). Therefore, the possibility exists that the M1 and M1–vRNP complex may be directed to the assembly site by a piggy-back interaction during exocytic transport of HA and NA (Ali et al., 2000). Furthermore, cytoskeletal components, particularly microfilaments, interact with the NP, vRNA of the vRNP and the M1–vRNP complex and thereby may facilitate the transport of these components to the assembly site (Avalos et al., 1997).

2.2. Interaction among the viral components

2.2.1. Interaction of M1 with vRNP and M1 with M1

Virus assembly and budding require that three subviral components, namely the viral envelope, M1 and vRNP, must interact with each other. Virion structure implies that M1 acts as a bridge between the envelope and the vRNP and therefore must interact with both the viral envelope on the outer side

and the vRNP on the inner side. As stated earlier, M1 was shown to interact with viral NEP (Akarsu et al., 2003; Ward et al., 1995; Yasuda et al., 1993), and vRNPs (Watanabe et al., 1996; Ye et al., 1999; Zvonarjev and Ghendon, 1980). The M1–vRNP complex can be isolated from either infected cells or purified virions by non-ionic detergent treatment under conditions where membrane glycoproteins are dissociated. M1–vRNP complexes are stable at neutral pH and low salt and can be dissociated only by high salt and acidic pH treatment (Zhirnov, 1992). However, the nature of M1–vRNP interaction is unclear at present since, as mentioned earlier, M1 does not interact with NP when expressed from cloned cDNAs (Zhao et al., 1998). It is likely that the M1–vRNP complex is formed by the interaction of M1 with the RNA of the vRNP. It has been shown that M1 interacts with the vRNP and inhibits transcription (Watanabe et al., 1996; Ye et al., 1987, 1999). Furthermore, M1 has been shown to bind ssRNA in vitro (Elster et al., 1997) and to vRNP in virus-infected cells (Lopez-Turiso et al., 1990; Ruigrok and Baudin, 1995) and in virus particles (Schulze, 1972), but M1 does not bind to NP expressed alone (Huang et al., 2001; Zhao et al., 1998). Also, the vRNA in the helical vRNP complex of influenza virus is exposed outside of the NP and is therefore available for interaction with M1. It was therefore postulated that M1 binds to the vRNP via negative charges on the exposed RNA in the vRNP (Baudin et al., 1994). Clusters of positive charges on the helix 6 (H6) domain (aa 91–105) are believed to interact with negative charges (PO^{4-}) of RNA. However, this view has been questioned by a number of workers since the C-terminal fragment (aa 165–254) of M1, which does not bind to RNA, can bind to the vRNP (Baudin et al., 2001; Ye et al., 1999) and the N-terminal fragment (aa 1–164) which binds RNA, does not bind the vRNP (Baudin et al., 2001; Watanabe et al., 1996). Furthermore, only the entire M1 (aa 1–252), which binds both RNA and the vRNP, causes transcription inhibition (Baudin et al., 2001). Interaction between the M1 protein and RNA was demonstrated by using filter-binding assays and blotting procedures (Wakefield and Brownlee, 1989; Ye et al., 1989). However, such artificial interaction of M1 with single stranded RNA is non-specific. Moreover, although the M1 protein contains a putative zinc finger motif, this motif is not involved in any biological function in virus replication in cultured cells (Hui et al., 2003b).

M1 interacts with itself and forms dimers and multimers (Zhao et al., 1998) and this interaction is expected to involve sequences in the M domain (Harris et al., 2001). M1–M1 interaction is critical in many aspects of virus budding, including concentration of viral components at the budding site, exclusion of host proteins from virions, formation of the asymmetry in lipid membrane at the budding site, initiation of membrane bending and bringing host components to the budding site for closure of virus buds.

2.2.2. Interaction of M1 with envelope proteins

As mentioned earlier, the position of M1 in the viral structure implies that M1 forms a bridge between the envelope proteins and vRNP and that M1 interacts with the envelope proteins, namely HA, NA, and M2, on the outer side. However, experiments to demonstrate direct interaction of HA and NA with M1 yielded contradictory results. Since a significant fraction of M1 alone was shown to bind membrane (Bucher et al., 1980; Gregoriades and Frangione, 1981; Hay, 1974; Ruigrok et al., 2000), coexpression of M1 with HA and NA did not significantly increase the membrane association of M1 (Kretzschmar et al., 1996; Zhang and Lamb, 1996). However, in one report, the HA and NA CTs have been shown to stimulate the membrane association of the M1 protein (Enami and Enami, 1996), and short synthetic peptides of the HA cytoplasmic sequence inhibited virus production (Collier et al., 1991). Subsequently, Triton X-100 (TX-100) detergent treatment at low temperature was used to demonstrate the specific interaction of M1 with both HA and NA (Ali et al., 2000). Both HA and NA were shown to associate with lipid rafts and become TX-100 resistant but M1 expressed alone was not raft-associated and was TX-100 soluble. However, when M1 was coexpressed with HA and NA, the membrane-bound M1 interacting with mature HA and NA became resistant to TX-100 either due to direct or indirect association of M1 with lipid rafts. Moreover, the interaction of M1 with HA and NA was shown to be specific for TX-100 resistance of M1 since the membrane-bound M1 in cells coexpressing M1 with a heterologous protein such as Sendai virus F protein, was not TX-100 resistant (Ali et al., 2000).

Furthermore, chimeras between HA and Sendai virus F proteins showed that both CT and the TMD of HA rendered the membrane-bound M1 resistant to TX-100, supporting the interaction M1 with both CT and TMD of HA. Analysis by confocal microscopy also demonstrated that in influenza virus-infected cells, a fraction of M1 was colocalized with HA both in the presence and absence of monensin (Ali et al., 2000). In the presence of monensin, an inhibitor of exocytic transport, HA was present predominantly in the perinuclear Golgi region and was absent from the plasma membrane. M1 was also more concentrated in the perinuclear region and less on the cell periphery, supporting colocalization of M1 and HA in the Golgi region of influenza virus-infected cells (Ali et al., 2000). Fractions of M1 and NP also colocalized in virus-infected cells (Avalos et al., 1997). These biochemical and morphological analyses demonstrated the interaction of M1 with lipid membranes, HA, NA, and the vRNP in influenza virus-infected cells.

3. Selection of the budding site

It is generally believed that viral glycoproteins determine the site of virus assembly and budding. This notion comes from the fact that viral glycoproteins accumulate at the site of virus budding even when expressed alone. For example, glycoproteins of viruses such as hepatitis B virus, bunyaviruses, coronaviruses, and others that bud from the internal sub-cellular organelles, possess intrinsic determinants

for the same sub-cellular localization as the site of virus budding (for review, see Hobman, 1993). On the other hand, for viruses budding from the plasma membrane, the viral glycoproteins possess either apical or basolateral sorting signals and are directed to the specific site where virus assembly and budding occur in polarized epithelial cells. Furthermore, in different cells and tissues where some viruses bud from the opposite domains of the plasma membrane, their glycoproteins are distributed accordingly. For example, Semliki Forest viruses (SFV) buds apically from FRT cells but basolaterally from CaCo-2 cells; similarly, in the absence of any other viral protein, p62/E2, the envelope glycoproteins of SFV, are targeted apically in FRT cells but basolaterally in CaCo-2 cells (Zurzolo et al., 1992). For retroviruses, particularly the human immunodeficiency virus (HIV) that buds from the basolateral surface in polarized epithelial cells, the HIV envelope protein is also directed basolaterally. HIV capsid proteins expressed alone released virus-like particles (VLPs) randomly from both apical and basolateral surfaces, whereas upon expression of the envelope protein gp160, particles were released predominantly from the basolateral surface (Owens et al., 1991). The authors concluded from these studies that the HIV envelope protein, which is targeted to the basolateral surface in polarized epithelial cells, determines the site of virus budding.

Influenza virus, which assembles and buds from the apical plasma membrane in polarized epithelial cells, has been used extensively as a model for studying protein targeting. Of the three transmembrane envelope proteins, HA is the major glycoprotein, comprising over 80% of the envelope proteins present in the virus particle. In transfected cells, a single amino acid change (Cys543 → Tyr543) in HA (HAtyr) was shown to direct HAtyr predominantly to the basolateral side without significantly affecting the intracellular transport and cell surface expression of the mutant protein (Brewer and Roth, 1991). Recently, using transfectant influenza virus containing basolaterally targeted HA (Cys543 → Tyr543), it was shown that the basolateral targeting of HA did not significantly alter the apical budding of influenza virus (Barman et al., 2003; Mora et al., 2002). Over 99% of the virus particles containing the HAtyr were released from the apical side even though the majority of HAtyr was directed to the basolateral side. However, the role of NA and M2 in polarized budding of influenza virus has not been examined yet. Similarly, when a mutant vesicular stomatitis virus (VSV) G protein was targeted apically, it did not affect the basolateral budding of VSV (Zimmer et al., 2002). It was also demonstrated that although measles virus glycoproteins H and F were transported in a random fashion or to basolateral membrane, respectively, virus budding occurred predominantly from the apical surface of polarized MDCK cells (Maisner et al., 1998). Similarly, although Marburg virus buds predominantly from the basolateral surface, its glycoprotein was transported to the apical surface (Sanger et al., 2001). These studies suggest that viral glycoproteins may not be the only or major determinant for selecting the site of virus budding and other viral components including M1 and vRNP as well as host components may be involved in determining the budding site.

4. Bud formation and completion

Budding requires the selection of an assembly site where viral components are transported and assembled leading to the initiation of the budding process, growth of the bud and finally, completion of the bud with the release of the virus particles. Each of the steps in the budding process is complex and requires involvement of both host and viral components. Influenza viruses not only bud from the plasma membrane, but they bud from the apical domain of the plasma membrane in polarized cells. In addition, influenza viruses do not bud randomly from the plasma membrane but discretely from preferred sites in the membrane. Alternatively, as proposed for murine leukemia virus (MuLV), budding of one virion may seed for the next in the same site and a defect in bud release could lead to joining of multiple particles forming filaments and this process may be coupled with the recruitment of host cytoskeletal elements at the preferred site of budding (Yuan et al., 2000). With influenza virus, cytoskeletal-disrupting agents caused an increased release of spherical over filamentous particles in MDCK cells (Roberts and Compans, 1998) and release of virus particles in a few localized regions of the plasma membrane in abortively infected HeLa cells (Gujuluva et al., 1994).

Bud formation and bud release are the last steps in viral replication and production of new infectious virions. Initiation of bud formation requires bending of membrane and involves a transition from more planar membrane structure to a curved structure (for review, see Farsad and De Camilli, 2003; Lippincott-Schwartz and Liu, 2003). Recently, a newly recognized BAR (Bin/Amphiphysin/Rsv) domain has been demonstrated to be involved in membrane curvature (Peter et al., 2004). This domain is present in a number of proteins involved in vesicle formation and recycling, such as amphiphysins, endophilins, arfaptins, nadrins, beta-centauins, and oligophrenins (for review, see Habemann, 2004; Lee and Schekman, 2004; Zimmebeg and McLaughlin, 2004). However, the role of any of these proteins in virus budding is unknown. Both lipids and proteins are likely to contribute to causing membrane curvature. Asymmetry in lipid bilayers can cause intrinsic curvature of one monolayer relative to the other monolayer leading to membrane bending (Holopainen et al., 2000). Therefore, assembly of lipid bilayers into specific lipid microdomains such as lipid rafts at the site of budding is likely to contribute to virus budding. In addition to specific lipid microdomains, virus bud formation requires specific viral proteins.

Two types of proteins that are associated with the viral envelope, namely (i) the transmembrane proteins HA, NA and M2 forming the outer spikes and (ii) the matrix protein M1 interacting with the inner leaflet of the lipid bilayer, appear to

play a critical role in budding. Clustering of M1 on the inner bilayer can cause membrane bending and initiation of budding. Finally, pinching off of the virus buds requires fusion of the apposing viral and cellular membranes leading to fission and separation of the bud from the cell (for review, see Nayak and Hui, 2004). Furthermore, influenza virus particles are pleomorphic. Although laboratory-adapted viruses are usually spherical, viruses freshly isolated from the field are generally filamentous. However, some laboratory-adapted strains (e.g. A/Udorn/72 [H3N2]) are also filamentous (Bourmakina and Garcia-Sastre, 2003; Roberts et al., 1998). Factors affecting the fusion of the lipid bilayers and fission of the bud will affect the size and shape of the virus particles. Among the viral components, M1 proteins have been shown to be the key component in both bud formation and pinching off (for review, see Nayak and Hui, 2002, 2004). In addition to viral components, a number of host components play a critical role in bud completion and virus release (for review, see Freed, 2002, 2003; Luban, 2001; Pornillos et al., 2002).

5. Role of M1 in virus budding

M1 is the most abundant protein in the influenza virion and plays critical roles in many aspects of the virus life cycle including virus budding (for review, see Nayak, 1996; Nayak and Hui, 2002). These include: (i) M1 interaction with vRNP and NEP and regulation of vRNP transport between the cytoplasm and the nucleus (for review, see Cros and Palese, 2003; Portela and Digard, 2002); (ii) regulation of vRNP transcription and replication; (iii) interaction with viral envelope proteins (HA, NA, and M2); (iv) recruitment of viral components at the assembly site and initiation of budding; (v) recruitment of host components for bud completion and virus release.

The M1 monomer is 60 Å long and possesses two globular regions (aa 1–164 and 165–252) linked by a protease sensitive loop. The structure consists mostly of helix and loops and is devoid of β-strands (Shishkov et al., 1999). The N-terminal fragment (aa 1–164) has been crystallized at both acidic and neutral pH and the 3-D structure has been determined by X-ray diffraction analysis (Arzt et al., 2001; Harris et al., 2001, 1999; Sha and Luo, 1997). This fragment contains eight loops (L) and nine helices (H) but the last loop (aa 159–164) was not resolved in the X-ray diffraction study. The H6 domain (aa 91–105) of M1 provides multiple functional domains including a nuclear localization signal, an RNA-RNP binding site, transcription inhibition motifs, and others.

M1 is the major driving force of influenza virus budding, since in the absence of M1 VLPs are not formed (Gómez-Puertas et al., 2000; Latham and Galarza, 2001). M1 aids in the assembly and budding process in multiple ways. M1 interacts with the inner leaflet of the lipid bilayer and thereby creates asymmetry in the membrane bilayer causing outward bending for the initiation of bud formation. M1 is believed to be the key protein in recruiting, concentrating, and assembling viral and host components required for budding at the assembly site of the plasma membrane (for review, see Lamb and Krug, 2001; Nayak, 2000; Nayak and Hui, 2002). M1–M1 interaction facilitates the formation of an M1 protein patch and the exclusion of host proteins from the assembly and budding site. M1 was shown to be a determinant for morphological shape and size (filamentous versus spherical) of influenza particles (Bourmakina and Garcia-Sastre, 2003; Hughey et al., 1995; Liu et al., 2002; Roberts et al., 1998). Since the M1 protein alone in the absence of any other viral proteins becomes membrane-associated (Ali et al., 2000; Kretzschmar et al., 1996) and produces VLPs in the extracellular medium (Gómez-Puertas et al., 2000; Latham and Galarza, 2001), the M1 protein has all the structural information needed for self-assembly, interaction with the plasma membrane, as well as initiation, completion and release of the bud. However, the interaction of M1 with both viral integral membrane proteins and newly assembled vRNP in the plasma membrane is believed to increase the efficiency of viral budding.

The matrix proteins of many negative strand viruses and the Gag proteins of retroviruses possess specific motifs called late (L) domains which are involved in recruiting the host components required for bud completion and virus release (for review, see Cimarelli and Darlix, 2002; Freed, 2002, 2003; Luban, 2001; Perez and Nolan, 2001; Pornillos et al., 2002; Yap and Stoye, 2003). So far, three different L domain motifs, namely PP(P/X)Y (PY motif or proline-rich motif), P(T/S)AP, and YP(D/X)L motifs have been found in the matrix proteins of negative strand viruses as well as retroviruses including HIV. These motifs have been shown to interact with a number of cellular proteins involved in bud completion (for review, see Freed, 2002, 2003; Luban, 2001; Perez and Nolan, 2001; Pornillos et al., 2002; Yap and Stoye, 2003). Recent studies using site-directed mutagenesis and rescuing mutated viruses by reverse genetics have shown that the helix 6 (H6) domain of influenza A virus M1 also possesses a L domain-like motif (Hui et al., 2003a). Mutation in H6 (R101A) was shown to reduce virus yield due to a budding defect producing filamentous particles (Fig. 1). The morphological phenotype of the R101A M1 mutant was strikingly similar to that observed for MuLV Gag mutants with L domain defects (Yuan et al., 2000). Both the R101A influenza virus mutant and the MuLV mutant exhibited elongated filamentous morphology. Many filaments contain multiple spherical units, a "daisy chain like structure" (Fig. 2B), suggesting a defect in releasing spherical particles during budding as was also seen with MuLV (Yuan et al., 2000) and other retrovirus L motif mutants (Garrus et al., 2001). Furthermore, the YRKL sequence of influenza M1 H6 domain can be replaced by a foreign L motif such as PTAP or YPDL but not by PPPY. Insertion of the YRKL into different locations of the mutated M1 protein restored normal budding but not the NLS function (Hui and Nayak, unpublished data). These results showed the interchangeable nature of the L domain motif of influenza virus M1. Taken together, these data indicate that

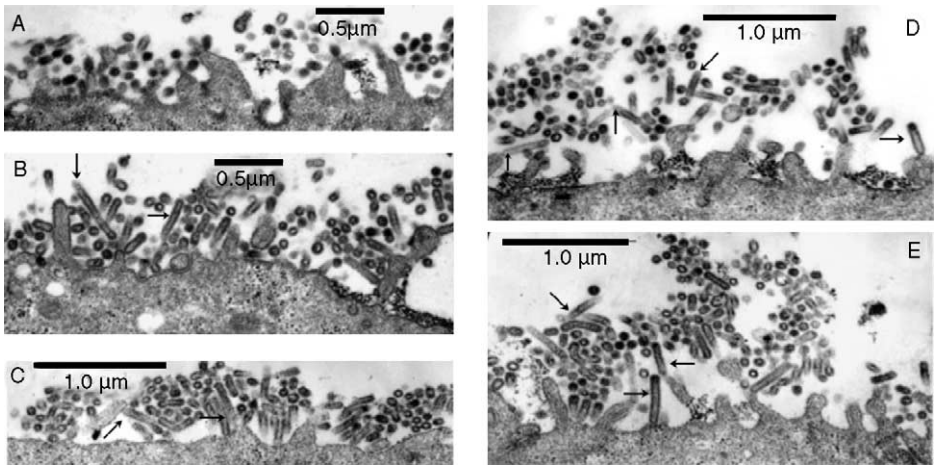


Fig. 1. Mutations in M1 and NA produce elongated influenza virus particles in MDCK cells. MDCK cells grown on polycarbonate filters were infected with different viruses at 3.0 MOI. At 12 h p.i., infected cell monolayers were examined by thin section electron microscopy. Results show that cells infected with an M1 mutant (R101A, panel B), and NA CT mutant (NA3A2, panel D) and NA TMD chimeras with TR [NATRNA and NA(1T2N)NA, panels C and E, respectively], produced elongated particles (→), whereas cells infected with WT virus produced mostly spherical particles (panel A). This figure was adapted from (Hui et al., 2003a) and (Barman et al., 2004) with the permission of American Society for Microbiology.

YRKL sequence and the neighboring region function as the L domain in influenza virus budding. Although the precise sequence and boundary of the L domain motif of influenza virus M1 are yet to be determined, the influenza L domain consists at least partly of the positively charged residues of the NLS sequence. Furthermore, since the Y and L of YRKL sequence can be replaced (Hui et al., 2003a), it does not represent a known L motif.

Like other viral L domains, the influenza virus L domain appears to function in bud completion rather than bud initiation and may be involved in recruiting host proteins required for bud completion and release of virus particles. In other viruses, the domains (P[T/S]AP, PP[P/X]Y, and YP[D/X]L) have been shown to interact with a number of host proteins involved in endocytic vacuolar sorting pathways such as Tsg101, Nedd4, ubiquitin ligases, AP2, and proteins containing SH3 and WW domains (for review, see Freed, 2002, 2003; Luban, 2001; Perez and Nolan, 2001; Pornillos et al., 2002; Yap and Stoye, 2003). However, host protein(s) interacting with the influenza virus L-like motif involved in budding have not yet been identified. It is likely that positively charged residues of the H6 domain may interact with a different set of host proteins which may be involved in apical budding whereas the PTAP and YPDL sequences may interact with proteins involved in basolateral budding.

5.1. Role of transmembrane proteins in virus budding

Among the three transmembrane viral envelope proteins (HA, NA, and M2), M2 is only a minor component (16–20 molecules/virion) and is therefore unlikely to play a significant role in budding. Moreover, recently it has been shown that the presence of M2 is not an obligatory requirement for virus replication. Infectious virus lacking M2 can be rescued and propagated in cell culture (Watanabe et al., 2001b). HA is the most abundant envelope protein (∼80%). However, HA may also have little role in the process of virus budding and release. Viruses lacking HA have been shown to release virus particles efficiently into the extracellular medium (Gómez-Puertas et al., 2000; Latham and Galarza, 2001; Pattnaik et al., 1986), although such particles are not infectious. HA lacking the CT did not cause aberrant virus budding or virus morphology (Jin et al., 1997).

On the other hand, several studies suggest that NA plays a critical role in virus budding (Jin et al., 1997). Although NA is not an absolute requirement for influenza viral morphogenesis (García-Sastre and Palese, 1995; Mitnaul et al., 1996), NA is clearly an important player in optimal virus replication. In cells infected with a mutant virus lacking NA, progeny viruses were not only aggregated on the cell-surface, but most of the virus particles exhibited an elongated morphology indicating a possible defect in the budding process (Liu et al., 1995). Moreover, elongated morphology of mutant virus particles suggests that the defect is in bud release rather than bud formation. Six amino acids of the NA CT are extremely conserved and are likely to play an important role in virus budding. Studies using tail minus HA (HAt^-) and NA (NAt^-) mutant viruses showed that NAt^- (Mitnaul et al., 1996) but not HAt^- (Jin et al., 1994) virus particles were elongated in shape. In addition, deletion of the CTs of both HA and NA led to formation of bizarre filamentous virus particles (Jin et al., 1997). The authors further observed that CT deletion caused a reduction in raft association and concluded that reduced raft association was responsible for the budding defects (Zhang et al., 2000). However, complete deletion of the CT of NA could cause structural perturbation leading to protein instability and reduced lipid raft association. Recent studies using mutational analysis of the transmembrane and cytoplasmic domains of NA have shown that some TMD as well as CT residues play critical roles in viral morphogenesis

including virus shape, size, and titer (Barman et al., 2004). When the extreme N-terminal three amino acids of NA were replaced with alanine, the mutant virus particles exhibited elongated shape (Fig. 1) although its lipid raft-association was normal. Similarly, NA/TR chimeras containing complete or partial replacement of the NA TMD with human transferrin receptor (TR) TMD also caused a budding defect producing elongated particles (Fig. 1). Therefore, it is likely that NA either directly or indirectly may have a role in the budding process independent of raft-association (Barman et al., 2004) (Fig. 1).

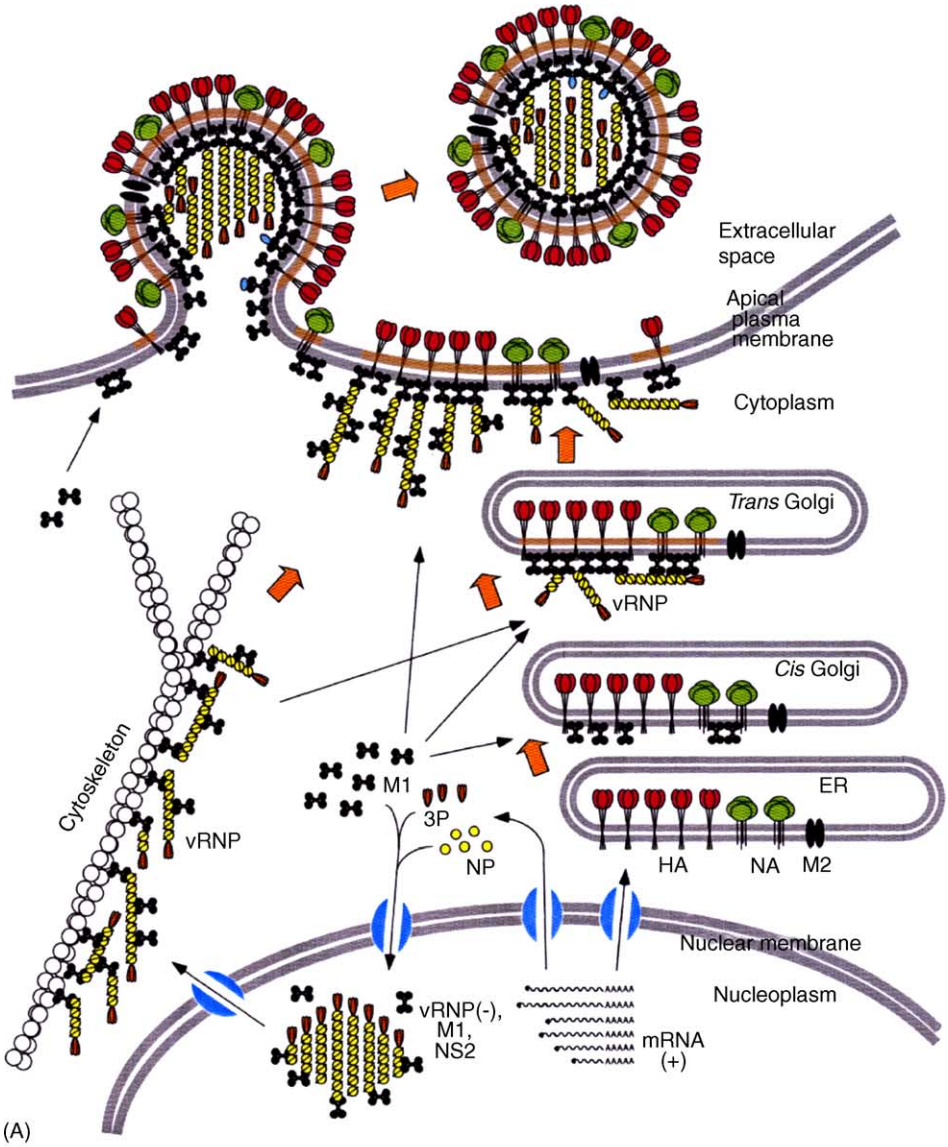
5.2. Role of the eight vRNP segments in virus budding

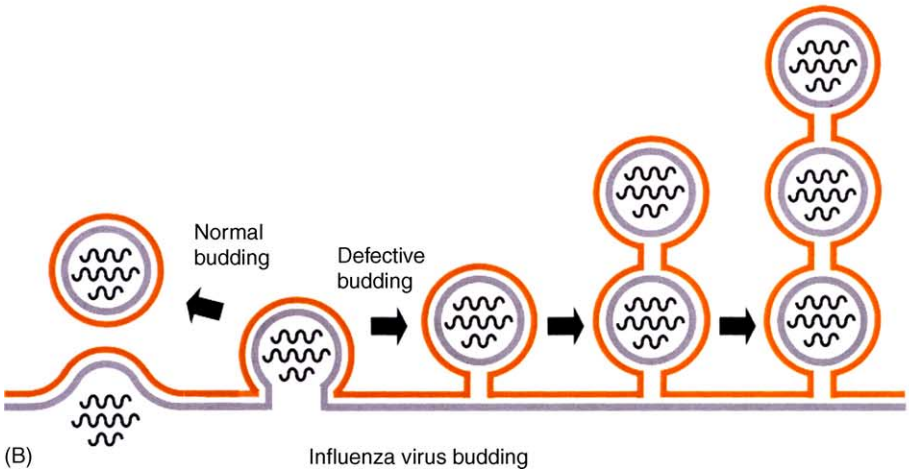
Although vRNP segments are not absolutely required for budding since M1 proteins alone can initiate and release virus buds (Gómez-Puertas et al., 2000; Latham and Galarza, 2001), incorporation of all eight (influenza A and B) or seven (influenza C) vRNA segments is required for the formation of infectious virus particles. However, how these multiple vRNA segments are incorporated into virus particles remains unclear. Two models have been proposed for the incorporation of eight vRNA segments into virions; i.e. "random packaging" and "specific packaging". The "random packaging" model predicts the presence of common structural elements in all vRNPs causing them to be incorporated randomly into virions. Support for this model comes from the observation that influenza A virions can possess more than eight vRNPs (9–11 vRNAs per virion) (Bancroft and Parslow, 2002; Enami et al., 1991). On the other hand, the "specific packaging" model assumes specific structural features in each vRNA segment, enabling them to be selectively incorporated into virions. Evidence for this model is deduced mainly from the finding that the various vRNAs are equimolar within viral particles even though their concentrations in infected cells may differ (Smith and Hay, 1982). Earlier studies demonstrating that the DI vRNAs can competitively inhibit the packaging of their normal counterparts but not that of other vRNAs argue for the specific packaging model (Duhaut and McCauley, 1996; Nakajima et al., 1979; Nayak et al., 1982, 1989; Odagiri and Tobita, 1990). Moreover, recent studies have shown that in addition to 5′ and 3′ non-coding sequences, specific coding sequences are required for efficient packaging of HA (Watanabe et al., 2003), NA (Fuji et al., 2003), M and NS genes (mentioned in Watanabe et al., 2003). Existence of specific packaging sequences would argue in favor of specific packaging over random incorporation of vRNA segments. Specific vRNA–vRNA interaction among the vRNP segments in *trans* would be involved in forming multi-segmental vRNA macromolecules for incorporation in virus particles. However, such a model would require that such large vRNP complexes containing eight unique vRNPs produced by RNA–RNA interactions in vRNPs in *trans* are stable. More importantly, bud closure and virus release will not occur until such vRNP complexes containing eight specific vRNP segments are formed. As mentioned earlier, there is as yet not evidence in support of specific vRNP segment requirement for bud formation and bud closure since M1 protein alone can form buds and release VLPs (Gómez-Puertas et al., 2000; Latham and Galarza, 2001).

5.3. Role of lipid rafts in virus budding

Viral morphogenesis is a complex phenomenon requiring concerted actions of many viral and host components (for review, see Cadd et al., 1997; Garoff et al., 1998; Nayak, 2000; Pettersson, 1991; Simons and Garoff, 1980). Among the host components that are intimately involved in regulating different aspects of the influenza virus life cycle, lipid rafts play a number of important roles. Lipid rafts are lipid microdomains enriched in sphingolipids and cholesterol. They contain lipids in liquid order (l_o) and are relatively resistant to non-ionic detergent at a low temperature (for review, see Brown and London, 1998; Simons and Toomre, 2000). Lipid rafts play critical roles in many aspects of the virus life cycle such as virus entry and uncoating, viral protein transport and targeting, selection of the viral assembly site, interaction among viral components, and finally, the budding process including bud initiation and bud completion (for review, see Barman et al., 2001; Chazal and Gerlier, 2003; Kielian et al., 2000; Nayak and Barman, 2002; Nayak and Hui, 2004).

Among the three influenza viral envelope proteins, both HA (type I) and NA (type II) proteins use lipid rafts as a platform for apical transport (for review, see Nayak and Barman, 2002; Nayak and Hui, 2004) but M2, although an integral membrane protein, does not use lipid rafts for apical transport (Zhang et al., 2000). Furthermore, in the envelope of released virions, both HA and NA remain raft-associated but M2 does not associate with the lipid rafts, indicating that the influenza viral envelope also exhibits a mosaic mixture of both raft and non-raft lipid microdomains even though the majority of lipids present in the lipid bilayer of the viral envelope are in the l_o phase. Association of HA with lipid rafts is not dependent on oligosaccharide modification or its association with other viral proteins or its assembly into virus particles. Neither does the association of HA with lipid rafts depend on the polarity of cells (Skibbens et al., 1989). The TMD of HA has critical determinants for interacting with lipid rafts since chimeric proteins containing the TMD of VSV G or HSV C proteins and the ectodomain and CT of HA did not associate with lipid rafts. Furthermore, the exoplasmic half of the HA TMD was critical for lipid raft-association (Lin et al., 1998; Scheiffele et al., 1997, 1999). In addition, palmitoylation of three cysteine residues present in the HA TMD and CT as well as structural features such as the α-helical conformation of the HA TMD peptide aid its interaction with lipid rafts (Melkonian et al., 1999; Tatulian and Tamm, 2000). Tail-minus HA (HAt^-) exhibited markedly reduced TX-100 resistance both in released virus particles and in cDNA transfected cells (Zhang et al., 2000). This could be partly due to the loss of two cysteine residues in the CT.


Extracellular space
Apical plasma membrane
Cytoplasm
Trans Golgi
vRNP
Cis Golgi
ER
HA
NA
M2
M1
3P
NP
Cytoskeleton
vRNP
Nuclear membrane
Nucleoplasm
vRNP(-), M1, NS2
mRNA (+)
(A)


Normal budding
Defective budding
(B)
Influenza virus budding

NA, a type II integral influenza virus protein, also associates with lipid rafts via its TMD during intracellular transport (Barman et al., 2001; Barman and Nayak, 2000). However, unlike HA, interaction of the NA TMD with lipid rafts was not dependent on acylation of cysteine residues. Like HA, the CT of NA affected the interaction of NA in the lipid raft since removal of the conserved CT reduced raft-association and increased TX-100 solubility of NA (Zhang et al., 2000).

Lipid rafts also play an important role in pseudotyping. Pseudotyping is a common phenomenon observed in cells co-infected with two or more enveloped viruses where progeny viruses containing the genome (and capsid) of one virus and the envelope proteins of a second virus are formed. This type of mixing of core (capsid) components with envelope components has been observed with many DNA and RNA viruses (Pickl et al., 2001). This well-documented phenomenon of pseudotyping is at odds with the common notion that specific interaction between the core component and envelope proteins governs the assembly and budding of most viruses. It appears that lipid rafts facilitate pseudotyping by forming a common platform for mixing the envelope proteins of different viruses and cellular membrane proteins. Even viruses belonging to diverse groups such as herpes simplex virus and VSV can produce pseudotyped viruses. A common property among all these diverse viral and cellular proteins are that they are raft-associated and often myristoylated or palmitoylated. The basolateral VSV G protein is TX-100 soluble indicating that it is not raft-associated. However, by antibody-induced patching experiment, it was shown that VSV-G partially co-patched with the raft-associated marker protein placental alkaline phosphatase (PLAP) indicating the partial raft association of VSV G (Harder et al., 1998). It was observed that in mixed infections the envelope proteins of different viruses as well as some of the core proteins such as HIV Gag are often raft-associated and non-ionic detergent insoluble (Pickl et al., 2001). Therefore, detergent-resistant lipid rafts on the plasma membrane are the common meeting ground for core and transmembrane envelope proteins of different viruses. Also, these lipid rafts function as a platform for envelopment and budding resulting in the production of pseudotyped viruses. Some of the host components such as CD4, CXCR4, as well as the envelope proteins of retroviruses and GPI-anchored proteins can be incorporated in the viral envelope. Lipid rafts therefore provide the basis for promiscuity in the incorporation of foreign proteins into a number of virus particles such as VSV, HIV, and influenza virus and support the passive incorporation of integral membrane proteins into virus particles. However, envelope protein and core interactions also play a critical role in selecting incorporation of specific viral proteins and in excluding most membrane-associated host proteins from the budding site and from virus particles. Therefore, lipid microdomains such as lipid rafts facilitate mixing and interaction among the viral components required for assembly and budding of infectious viruses as well as in pseudotype formation.

Lipid rafts, in addition to transporting, targeting and concentrating viral and host components to the assembly site, may have some intrinsic properties for initiating budding and thereby facilitate budding from specific sites of the membrane for a given virus. Raft association of HA appears to be responsible for clustering of HA on the plasma membrane and efficient budding but has no effect on virus morphology (Takeda et al., 2003). There are a number of reasons why different viruses choose different lipid microdomains for budding. Influenza virus HA and NA associate with lipid rafts, and influenza viruses bud from lipid rafts. The presence of specific peptides in a specific conformation often facilitates association with lipid rafts and may increase the order of lipids in the lipid raft. For example, the helicity of the HA TMD peptide increased in lipid bilayers composed of acidic lipids and in turn, the presence of the peptide also increased the acyl chain order of the lipid bilayer. Ordered lipids attract TMDs and TMDs in turn increase the order of the lipids surrounding them. This process may aid in targeting HA and NA transmembrane proteins to ordered lipid rafts and organizing ordered lipid rafts around them (Tatulian and Tamm, 2000). However, incorporation of HA alone is not sufficient to organize an ordered lipid environment since HA incorporated in the VSV envelope is TX-100 soluble (Scheiffele et al., 1999). Furthermore, raft-dependent protein–protein interactions may facilitate bringing proteins that are present in less-ordered membrane to lipid rafts by interaction with raft-associated proteins. Interaction between influenza virus M1 and HA brings M1, a non-raft-associated protein, into lipid rafts (Ali et al., 2000). Also, raft-ordered membrane domains may be formed de novo around transmembrane proteins on

Fig. 2. (A) Schematic representation of influenza virus morphogenesis. For viral morphogenesis to occur, all the subviral components must be transported to the assembly site at the plasma membrane and interact with one another in an orderly manner. Both glycoproteins (HA and NA) use the exocytic pathway and are transported from the *trans* Golgi network to the budding site, a specific region on the plasma membrane containing lipid rafts. Another glycoprotein (M2), is transported via the same route but does not require lipid rafts. The Ml–vRNP complex, consisting of the viral genomic RNA, NP, NEP, 3P and Ml, are exported out of the nucleus and are transported to the assembly site on the plasma membrane either via cytoskeleton elements or by piggy-backing on the cytoplasmic tail of HA and NA. Ml binds to the cytoplasmic tail and transmembrane domain of HA and NA on the outer side, and the vRNP on the inner side. Finally, the plasma membrane bends at the assembly site containing glycoproteins and the Ml–vRNP complexes, causing an outward membrane curvature. Eventually, fusion of the apposing cellular and viral membranes leads to fission and pinching-off of the virus particle, releasing the enveloped progeny virus particle into the extracellular medium. Lipid raft microdomains in the membrane are shown in brown; non-raft regions are depicted in grey. (B) Schematic representation of defective and normal virus budding. Mutant viruses with defective budding produce structures joining multiple particles (Hui et al., 2003a) due to incomplete fusion and fission of apposing cell and viral membranes. The presence of multiple incomplete virus-like particles in elongated structures suggests that virus budding from the plasma membrane is not random but occurs at specific sites, producing multiple virus particles from the same site. Although three segments are depicted, each influenza particles contain eight or more vRNP segments.

the plasma membrane such as the engaged immune receptors (for review, see Cheng et al., 2001; Mañes et al., 2001). The affinity of l_o domains can be increased by organization, acylation, coupling to raft-associated molecules or by conformational changes (Harder et al., 1998).

Although viruses can bud and form particles (VLPs) in the absence of glycoproteins and although the Gag protein of HIV and the matrix proteins of many negative strand viruses can bud and acquire envelope, the lipid composition of such VLPs is not known. Whether these VLPs contain lipid rafts in their envelope or whether glycoproteins are required for acquiring lipid rafts in their envelope remains to be determined. The lipid composition of a VLP's envelope may indicate whether virus budding occurs from the plasma membrane outside lipid raft microdomains or whether budding requires the presence of lipid raft microdomains.

Finally, involvement of lipid rafts in virus replication may provide a novel antiviral approach. Topical application of β-cyclodextrin (β-CD), a lipid raft destabilizer, shows the promise of antiviral effect in HIV transmission (Khanna et al., 2002), possibly by preventing virus entry or by disrupting virus budding or virion structure.

5.4. Role of host proteins in virus budding

In addition to lipids, a number of host proteins, including microfilaments, G proteins, and some protein kinases, have been shown to be involved in the budding of many enveloped viruses (for review, see Ludwig et al., 1999). In addition, the family of proteins of the vacuolar protein sorting pathway have been shown to interact with the L domains of the Gag and matrix proteins of a number of viruses. These include Tsg101, other ESCRT components, and proteins containing WW domains (such as Nedd4 family proteins) (Garrus et al., 2001; Martin-Serrano et al., 2004; Ono and Freed, 2004; Strack et al., 2000, 2003; von Schwedler et al., 2003). However, for influenza viruses, host protein(s) that interact with M1 and specifically affect virus budding have not yet been identified. Furthermore, inhibitors of proteasomes involving ubiquitination were found to inhibit budding of a number of enveloped viruses (for review, see Vogt, 2000) although the specific role of ubiquitination in virus budding remains unclear. However, inhibitors of ubiquitination did not affect influenza virus budding (Hui and Nayak, 2001). Cytoskeletal elements, particularly microfilaments, have been proposed to be involved in the maturation of influenza virus including bud formation and bud completion. In abortively influenza virus-infected HeLa cells, virus particles could be released using microfilament-disrupting agents (Gujuluva et al., 1994). Also, the budding of filamentous influenza virus particles was converted to spherical particles by inhibitors of actin polymerization such as cytochalasin B (cytoB), cytochalasin D (cytoD), jasplakinolide, and latrunculin A (Roberts and Compans, 1998; Simpson-Holley et al., 2002), suggesting the role of actin microfilaments in bud formation and bud release. In addition, the vRNP–M1 protein complex in virus-infected cells as well as NP but not M1 alone in cells expressed from cloned cDNA interacted with F-actin (Avalos et al., 1997; Bucher et al., 1989; Digard et al., 1999, 2001; Husain and Gupta, 1997). Furthermore, actin was found in many enveloped virus particles (for review, see Cudmore et al., 1997; Eaton and Hyatt, 1989; Falke, 1997; Wang et al., 1976). Also, actin and actin-binding protein ezrin-radixin-moesin (ERM) have been found in influenza virus particles (Sagara et al., 1995). The presence of actin-associated proteins in virions suggests specific functions of the actin filament during assembly and budding.

Influenza virus budding was shown to be an active, energy-dependent process requiring ATP hydrolysis (Hui and Nayak, 2001). Metabolic inhibitors (such as antimycin A, CCCP, FCCP, and oligomycin) and ATP analogues (such as ATPγS and AMP-PNP) inhibited influenza virus budding (Hui and Nayak, 2001). Energy is required for biomembrane bending and shape transition during bud formation (Sackmann, 1994). ATP may play a multifunctional role during influenza virus budding by maintaining a lipid raft membrane structure favorable for virus budding, by providing the energy for membrane shape transition or actin polymerization, and by functioning as a molecule for protein kinase signaling during virus budding.

Among the kinases, casein kinase 2 (CK2) is involved in influenza virus budding since a CK2 inhibitor disrupted virus budding, and increased CK2 activity correlated with the replication cycle of influenza virus (Hui and Nayak, 2002). Moreover, CK2 was found in influenza virus particles (Tucker et al., 1990) suggesting its presence in the vicinity of the budding area of influenza virus and active involvement in the budding process (for review, see Hui, 2002, in press). However, inhibitors of cAMP-dependent protein kinase (PKA), protein kinase C (PKC), and phosphatidylinositol 3-kinase (PI3K) did not affect influenza virus budding. Furthermore, although the M1 protein is a phosphoprotein and both phosphorylated M1 and NP have been found in virus particles (Gregoriades et al., 1984, 1990), there is no evidence for a specific requirement for phosphorylation of any viral protein in the budding process.

5.5. Bud completion

Subsequent to bud formation, buds are released by a mechanism of fusion of the apposing membranes and fission of the bud from the cell membrane (Fig. 2B). These processes determine the size and shape of the particles. The mechanism of bud completion is yet unclear and a number of factors both viral and host may affect this process. For some viruses, such as Semliki Forest virus, the icosahedral nucleocapsids determine the spherical shape of the released virus particles. Similarly, the length of the helical VSV nucleocapsid is critical in determining the bullet shape and the length of the virus particles. Defective interfering VSV particles contain smaller nucleocapsids, which are responsible for producing small virus particles. Therefore, with these viruses, separation of virus

buds from host membranes depends on the cargo nucleocapsid and occurs immediately after the enclosure of the nucleocapsid. However, many viruses such as influenza are flexible and pleomorphic and can produce spherical or filamentous particles. With these viruses, a number of factors may play critical roles in causing the fusion and fission processes and determining the size and shape of the released virus particles.

As mentioned earlier, among the viral components, matrix proteins as well as glycoproteins have been shown to affect virus shape and size. Deletion of the CT of both HA and NA was shown to generate bizarre filamentous virus particles. Reduced lipid raft-association of HAt^{-}/NAt^{-} virus was proposed to be the cause of such abnormal virus particles suggesting the role of lipid rafts in both budding and fission of virus particles (Zhang et al., 2000). In addition, mutation in the CT of NA was shown to generate spherical to filamentous form not dependent on lipid raft association (Barman et al., 2004). With some influenza virus strains (A/Udorn/72) that exhibited filamentous morphology, M1 contributed to the strain-specific filamentous shape (Bourmakina and Garcia-Sastre, 2003; Roberts and Compans, 1998). Also, as mentioned earlier, influenza virus M1 possesses L domain activity that affects fission of virus buds (Hui et al., 2003a).

In addition to viral factors, a number of host proteins as indicated earlier (Section 5.4) including ubiquitin, Tsg101, Vps4, Nedd4, and other members of the vacuolar protein sorting pathway have been shown to be involved in the budding process (for review, see Freed, 2002, 2003; Luban, 2001; Pornillos et al., 2002; Yap and Stoye, 2003). All of these host proteins in some way facilitated the fusion and fission processes in bud release so that any defect in the interaction of these virus and host components led to defective or incomplete virus release, often forming multiple VLPs joined together. However, how these host proteins or their interactions with viral late domains facilitate the process of fusion and fission remains unclear. These defective particles were not completely filamentous or tubular but exhibited clover leaf-like or tethered structures (Garrus et al., 2001) suggesting incomplete membrane fusion and fission. Similar structures representing defective budding by joining of multiple particles due to incomplete fusion and fission were found in mutant influenza viruses (Hui et al., 2003a) (Fig. 1). It will be interesting to determine if these particles represent a state similar to hemifusion in which only the inner leaflet undergoes fusion and therefore cannot undergo complete fission and release from the host membrane and separation from each other (Fig. 2B).

In addition, as indicated earlier, cytoskeletal components, particularly actin microfilaments, have been shown to contribute to filamentous forms of influenza virus particles (Roberts and Compans, 1998). Microfilaments that bind to the vRNP may provide outward pushing force in bud formation. However, if actin is involved in the budding process, the fusion of membrane at the stalk of the bud and fission of buds will require disassembly of actin filaments at the last stage of the budding process. Enhanced release of virus particles from influenza virus-infected HeLa cells with actin disrupting agents (Gujuluva et al., 1994), as well as increase in spherical over filamentous particles in influenza- and parainfluenza virus-infected polarized MDCK cells (Roberts and Compans, 1998), support the role of actin depolymerization in bud closure.

Finally, lipid rafts can affect both bud formation and fusion and fission processes at multiple steps. As indicated earlier, asymmetry in the lipid bilayer can cause membrane curvature leading to the formation of buds (Holopainen et al., 2000) and reduced lipid raft association causes deformed virus particles (Zhang et al., 2000). Assembly of lipid rafts at the budding site will affect physical properties of the membrane including lipid heterogeneity, lipid–protein interaction, increased viscosity and rigidity, slow diffusion, etc. The presence of lipid heterogeneity could cause increased fission and release of buds. However, a specific role of lipid rafts in bud completion remains undefined.

6. Release of virus particles

Lastly, after their budding from the host cell, viruses must be released into the surrounding medium to infect other cells. With influenza viruses, bud formation and bud closure causing pinching off of the virus particle may not be sufficient to release the virus into the external environment since the released particles may still be attached to the infected host cell via sialic acid. The data from ts viruses at restrictive temperature, deletion or mutations of NA gene leading to the loss of NA enzyme activity as well as inhibitors of NA clearly demonstrate that NA activity is involved in virus release (Barman et al., 2004; Palese and Compans, 1976; Palese et al., 1974). The NA removes sialic acid, the receptor for influenza virus, from the membrane glycolipids and glycoproteins of both the virus particles and virus-infected cells and thus prevents self-aggregation of virus particles and reattachment to the virus-infected cell. However, as indicated earlier, NA is not critically required for the infectious cycle in cultured cells provided sialidase is present in the medium (Liu et al., 1995).

7. Role of virus budding in pathogenesis

In a natural setting of viral infection, either the human or animal host is infected at a very low MOI with relatively few virus particles. Therefore, multiple cycles of replication leading to release of new progeny viruses and infection of new host cells by the progeny viruses must be repeated many times and are critically required not only for the survival of the virus and cell-to-cell spread but also for producing the disease syndrome in the infected host. In most cases, viruses must kill, destroy or alter the function of a large number of cells of a specific organ or tissue before the specific functional abnormality in the form of a disease syndrome such as pneu-

monia, hepatitis, or acquired immune deficiency syndrome (AIDS), etc. is manifested. The site and the nature of budding can be an important contributory factor in viral pathogenesis particularly for respiratory viruses like influenza viruses. Influenza viruses bud from the apical surface of polarized epithelial cells (e.g. bronchial epithelial cells) into the lumen of lungs and are therefore usually pneumotropic, i.e. restricted to lungs, and do not cause viremia or invade other internal organs. However, some influenza viruses like fowl plague (H5 or H7) as well as WSN (H1N1) viruses (H1, H5, H7 indicate the HA subtype specificity of type A influenza viruses) are not restricted to lungs and produce viremia infecting other internal organs (pantropism) and cause severe mortality in infected animals (Mori et al., 1995; Subbarao and Katz, 2000). In humans, most influenza viruses are pneumotropic and do not spread to other internal organs. Why the Spanish flu virus of 1918 caused such a devastating pandemic, killing 20–40 million people world-wide and affecting young healthy adults, remains unclear. In addition to pneumonia, some people died due to massive pulmonary hemorrhage and edema. The 1918 Spanish flu virus, like fowl plague viruses, may have been pantropic causing viremia and infecting other organs. Why some influenza viruses are pneumotropic while others are pantropic and highly virulent is not fully understood. The severity of viral pathogenesis depends on both viral factors and host factors including host defense and immunity. Determinants for virulence of influenza viruses are complex and multigenic. However, as indicated earlier (see Section 1), one single factor critically required for viral growth and virulence is cleavability of HA → HA1 and HA2. Normally, influenza virus is restricted in lungs because its HA can be cleaved by *tryptase Clara*, a serine protease restricted to the lungs (Kido et al., 1999b). However, HAs of H5 and H7 pantropic avian virus subtypes contain multiple basic amino acids at the junction of HA1 and HA2 and can be cleaved by furin and subtilisin-type enzymes (Horimoto and Kawaoka, 1995), which are present ubiquitously. Such viruses can therefore grow in other organs. In addition, the NA of some influenza viruses like WSN binds and activates plasminogen into plasmin in the vicinity of HA and the activated plasmin cleaves HA → HA1 + HA2 rendering the virus infectious. Therefore, WSN virus lacking multiple basic residues in its HA can grow and multiply in tissues other than lungs. However, the sequences of HA and NA genes from the 1918 pandemic human virus, and predicted HA and NA protein amino acid sequences (Reid et al., 1999, 2000) cannot explain the severity of its virulence. It is likely that other viral genes are involved in the virulence of 1918 "Spanish" flu viruses (for review, see Reid and Taubenberger, 2003; Taubenberger et al., 2000). The presence of the WSN NA gene alone, which is responsible for HA cleavage, could not cause pantropism and neurovirulence in mice (Ward, 1995). Other WSN genes, like M, NS and NA, were required to cause neurovirulence (Schlesinger et al., 1998; Ward, 1996) and therefore were also required for viremia and pantropism. The specific function of M and NS genes in pantropism and neurovirulence remains unknown. The NS1 protein, an interferon antagonist, can contribute to virulence in a species-specific manner (Krug et al., 2003). As indicated earlier, the M gene, encoding M1 and M2 proteins, can affect virus replication at multiple stages of the infectious cycle and has a profound effect on virus virulence. However, the role of the M gene in the virulence of specific virus strains like the 1918 influenza virus is unknown. Recent studies with M1 mutants have shown that the M1 gene can have a profound effect on virulence of WSN virus in mice but no effect on virus replication and growth in MDCK cells in culture (Hui, Smee and Nayak, unpublished data). In Sendai virus, the M gene was shown to cause enhanced basolateral budding and increased virulence. Sendai virus mutant F1-R, which exhibited pantropism, possessed two characteristics: (i) like the H7, H5 HA, ubiquitous cleavage of mutant F → F1 + F2 due to the presence of multiple basic residues and (ii) altered budding from both the apical and basolateral surface possibly due to mutations in the M protein which caused disruption of microtubules and polarized transport (Tashiro et al., 1993, 1996). Therefore, altered budding could be an important contributing factor in the dissemination of virus into blood, invasion of internal organs, pantropism and consequently, higher virulence of a specific influenza virus strain.

8. Conclusion

Influenza viruses bud from the plasma membrane, more specifically from the apical domain of the plasma membrane in polarized epithelial cells both in vivo and in tissue culture. Assembly and morphogenesis of influenza viruses require the transport of the viral components to the assembly site and interaction among the viral components. Furthermore, influenza viruses bud from the apical plasma membrane and from specific membrane microdomains called lipid rafts present on the plasma membrane. Virus morphogenesis also requires an outward membrane curvature at the assembly site leading to bud formation, eventual fusion of the apposing membranes, fission of buds and separation of virus particles from cellular membranes, and virus release to the outside environment. These budding processes are active and energy-dependent, and are affected by physical factors such as membrane fluidity and viscosity at the budding site. Elucidation of the processes involved in the assembly and morphogenesis of virus particles is critical to understanding virus growth and multiplication is therefore crucial in defining viral infectivity, transmission, virulence, tissue tropism, host specificity and pathogenesis, and will contribute to an overall understanding of the disease process and progression of disease including morbidity and mortality of infected hosts. In addition, the site of budding can also affect virus virulence and pathogenesis. In this review, we have discussed the critical steps required for the assembly and morphogenesis of influenza viruses, i.e. directing the viral components to the assembly site and interactions among the viral components,

bud formation, closure of buds and release of virus particles. However, virus budding is among the least understood processes in virus biology and requires concerted action by a number of viral and host factors. Little is known about the host factors involved in influenza virus budding. A better understanding of viral replication and morphogenesis will facilitate the development of novel therapeutic agents capable of interfering with these critical steps in viral multiplication, pathogenesis and disease progression.

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